Ecology of gut bacteria – microbial community dynamics investigated using an integrated approach

Pål Trosvik

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
Nr. 775

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

ACKNOWLEDGEMENTS

First I would like to thank my supervisors at Matforsk, Knut Rudi and Tormod Næs, and at UiO, Nils Chr. Stenseth and Kjetill Jakobsen, for all their help in making this thesis become a reality. I would also like to thank the technical staff at Matforsk for excellent assistance in the lab. I am grateful to all the collaborators on my papers for their important contributions, and to my colleagues at Matforsk and CEES for providing an inspiring working environment.
I am also much obliged to my family and friends for their support during this period, in particular to my mother for reading the thesis (which I am sure was not 100% entertaining). I am extremely grateful to Eli for supporting and advising me these past few years.
Finally, I would like to thank the late Peder Haugen for his friendship and collaboration up through our study years.

LIST OF PAPERS:

Paper I)

Multivariate analysis of complex DNA sequence electropherograms for high-throughput quantitative analysis of mixed microbial populations.
Trosvik P, Skånseng B, Jakobsen KS, Stenseth NC, Naes T, Rudi K.
Appl Environ Microbiol. 2007 Aug;73(15):4975-83.

Paper II)

Co-infection dynamics of a major food-borne zoonotic pathogen in chicken.
Skånseng B, Trosvik P, Zimonja M, Johnsen G, Bjerrum L, Pedersen K, Wallin N, Rudi K.
PLoS Pathog. 2007 Nov;3(11): e175.

Paper III)

Characterizing mixed microbial population dynamics using time-series analysis.
Trosvik P, Rudi K, Næs T, Kohler A, Chan KS, Jakobsen KS, Stenseth NC.
ISME J. 2008 Jul;2(7):707-15. Epub 2008 Apr 3.

Paper IV)

Web of interactions in an experimental gut microflora.
Trosvik P, Rudi K, Strætkvern KO, Jakobsen KS, Næs T, Stenseth NC
Submitted to Proceeding of the Royal Society B

TABLE OF CONTENTS

INTRODUCTION

Microbial ecology

Prokaryotes (bacteria and archaea) are found in virtually every habitat imaginable on earth, from hot springs and deep-sea hydrothermal vents to soils and open oceans. These microbes form the basis of the planet's major biogeochemical cycles, and their activities up through the history of the earth have had profound impacts on practically every aspect of its environment (1). The estimated total number of prokaryotic cells in the biosphere is 4-6 x 10^{30}, amounting to 3.5-5.5 x 10^{14} kg of cellular carbon, an amount comparable to that of plants, and they constitute the largest living reservoirs of nitrogen and phosphorus on the planet (2). Broadly stated, the scope of microbial ecology is the investigation of how these organisms interact with each other and the environment.

In the course of evolution microbes have also formed many intimate relationships with multicellular organisms. Several species of fish, squid and jellyfish have bacteria in their tissues, enabling them to bioluminesce (3-5), tube worms and mussles living around deep water hydro-thermal vents depend on chemolithotrophic bacteria to provide them with nutrition (6), and the gut microflora of ruminants and termites allow these animals to subside on otherwise indigestible diets (7, 8). Humans also have comprehensive and important symbiotic relationships with bacteria, the extents of which are still not clear to us (9-11).

Microbiota of the gastroinstestinal system

The gastrointestinal (GI) tract of humans is home to a prolific community of bacteria. It is an extremely densely populated system with bacterial abundances as high as 10^{12} cells per ml of luminal content in the colon (2). Indeed, the bacterial cells in the human gut may outnumber human somatic and germ cells by more than one order of magnitude (12). According to current estimates the GI microbiota is composed of >500 species, depending on the criteria used for species delineation, and several thousand strains (13). The dominant phyla are Firmicutes and Bacteroidetes, comprising >90% of the normal adult human GI microbiota. Other phyla represented, in appreciable quantities, are Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia (14). Scientists are just

beginning to unravel the variety of important functions microbes contribute to host physiology, much thanks to the development of molecular methods (15) and suitable model systems (16). Examples of microbial contributions to host health include development and modulation of immunity (17-20), induction of intestinal angiogensis (21) and enhancement of energy metabolic capabilities (22-24). The human gut microbiota has also been implicated in a number of pathologies, examples of which are allergies (25, 26), colon cancer (27), inflammatory bowel disease (28) and obesity (29, 30). Its importance in health issues has made the GI microflora the focus of much research, which has led to radical changes in how we view this ecosystem.

Molecular methods in microbial ecology

Historically, microbiological research has largely been based on pure culture studies. This approach imposes obvious limitations on the amount of data that may be extracted from environmental samples, and the realization that microbes, in general, do not exist in pure cultures, prompted the development of alternative, culture-independent techniques in microbial ecology. Drawing on the molecular phylogenetic approach taken by Carl Woese and coworkers in the 1970s (31, 32), in the mid 80s Norman Pace and his group started employing nucleic acid based methods, focusing on ribosomal RNA genes, for the study of microbes in environmental samples (33-35). This development was accelerated by the discovery and increased use of the polymerase chain reaction (PCR)(36), and the early 1990s saw a great number of microbial ecology publications using molecular techniques (37-44), leading to a vastly increased appreciation of microbial diversity in the biosphere (45-48).

The most frequently used molecular marker for the exploration of microbial communities is the gene encoding the small subunit (SSU) ribosomal RNA molecule, also known as the 16S rRNA gene (47, 49). This gene occurs in all prokaryotic organisms, it has enough sequence variability to distinguish between a wide range of species, and it possesses conserved regions suitable for the application of universal PCR primers, allowing for broad range simultaneous amplification of 16S rRNA genes from environmental samples.

Most of the current molecular methods used in microbial ecology can be divided roughly into 3 categories, and combinations of these categories of techniques have also been in frequent use (50-52).

1. Community fingerprinting

Fingerprinting techniques use the differential migratory properties of DNA fragments during gel electrophoresis for describing microbial diversity in environmental samples. A few popular techniques separate PCR-amplified DNA fragments on the basis of sequence dissimilarities rather than length polymorphisms, producing community characteristic gel patterns. This can be achieved by applying denaturing gradients during one dimensional electrophoresis, either by the use of temperature or chemicals (53-55). A similar approach is single-strand-conformation polymorphism (SSCP) analysis which separates DNA fragments of equal size according single strand secondary structure (56, 57).

A second group of techniques uses length polymorphisms in PCR-amplified DNA fragments to produce microbial community fingerprints. One popular method is terminal-restriction fragment length polymorphism (T-RFLP) analysis, which depends on fluorescently 5'-end labeled 16S rRNA gene amplicons from different microbes being differentially digested by restriction endonucleases (58, 59). Thus, a particular sample will have a characteristic pattern of fluorescence peaks upon capillary electrophoresis.

Another approach within this group is rRNA intergenic spacer analysis (RISA) (60, 61). This method takes advantage length polymorphisms in the spacer region between the 16S and 23S rRNA genes of bacteria. Like T-RFLP analysis, RISA uses fluorescence tagged primers for PCR, but the primers target conserved genetic regions adjacent to the spacers. Similarly to T-RFLP analysis this produces a community specific peak pattern upon electrophoresis. RISA is also amenable to automation.

2. Hybridization-based techniques

Some techniques in microbial community analysis use hybridization between homologous DNA sequences in order to identify and enumerate microbes in community settings. These techniques have the advantage of providing a possibility for skipping the initial step of PCR amplification. Fluorescence in situ hybridization (FISH) uses labeled

DNA probes with a certain amount of taxonomic specificity in order to visualize the distribution of, as well as to quantify, one or more groups of microbes in complex communities (62-64).

DNA microarrays found their way into microbial ecology in the mid 90s (65), and the popularity of this approach has been steadily increasing (66). This technology offers the possibility of investigating both microbial diversity and abundance, using phylogenetic oligonucleotide arrays (52, 67). However, microarrays have also been used as functional gene arrays (FGAs) in order to detect certain metabolic pathways in environmental samples (68, 69).

3. DNA sequencing techniques

The perhaps most widely used technique in microbial ecology has been sequencing of environmental clone libraries. The traditional target for such studies has been PCR-amplified 16S rRNA genes. This was the approach first taken in the early 90s, and it has since been applied to a large variety of microbial ecosystems (14, 70-76).

Since its invention in the mid 70s, Frederick Sanger's chain termination method (74) has been the dominant DNA sequencing technology. Further development of this methodology (77, 78), along with the development of techniques allowing for large fragment cloning, such as bacterial artificial chromosome (BAC) vectors (79-81), set the stage for environmental genomics science, also known as metagenomics. This approach allows researchers to describe not only the phylogenetic diversity of microbes in natural environments, but also the genetic and metabolic diversity, without the need for prior cultivation (81-86).

The implementation of more recent technologies has vastly reduced the costs of DNA sequencing, while achieving equally vast increases in throughput (87-90). For example, emulsion PCR coupled with massively-parallel pyrosequencing, as implemented in the 454 sequencing technology, allows for the extremely rapid determination of microbial genome size DNA sequences (88). Although single sequence reads are shorter than for traditional Sanger sequencing, such technologies have been used in microbial ecology, both in phylogenetic (91, 92) and metagenomic (8, 30, 93) contexts.

Ecological theory in microbial ecology

Understanding how ecological principles operate to determine the structure and function of microbial systems presents a great challenge to microbiologists and ecologists alike. Given the huge numbers and diversity of microorganisms in the biosphere, the pivotal roles these organisms play in shaping the earth's environment, and their medical importance, this knowledge is of potentially great value. An important step towards expanding our knowledge of microbial community ecology is the implementation of ecological theory in this type of research. The bulk of the comprehensive body of ecological theory currently in existence has been developed by general ecologists using observations of more familiar animal and plant systems (94). Some studies of microbial systems, like the works of Gause in the 1930s (95, 96), have contributed to the development of some of the basic tenets in theoretical ecology, but in general, microbial systems have not been popular objects for ecological studies (97). This can, at least in part, be attributed to the difficulties inherent in observing microbes in complex systems, making collection of quantitative population data quite hard. However, there has also been a historical divide between the fields of general and microbial ecology, and in spite of their seemingly common goals, they have developed more or less independently of one another. Thus, much of the existing ecological theory has yet to be implemented in a microbial context (98). The traditional approach to microbiology has very much been one of reductionism, focusing mainly on the detailed description of physiological mechanisms. A trend toward descriptive studies can also be seen in microbial ecology, a development which has been greatly facilitated by the availability of cultivation-independent methods (8, 14, 54, 58, 71, 75, 76, 91, 93, 99). While having their own scientific merit, general ecological aspects are often lost from such studies. Providing snapshots of microbial community phylogenetic and metabolic structures, they tend to neglect fundamental community phenomena like temporal dynamics, species interactions and system stability. Such phenomena have long been major focus areas in general ecology, and by combining modern molecular techniques with existing ecological methods, researchers should now be able to address similar questions in microbial ecology.

As model systems for ecological studies microcosms also have a lot to offer. The short generation times of microbes facilitate data collection on time scales that are of evolutionary relevance, providing a unique opportunity for the investigation of interplay between ecology and evolution (100-102). Furthermore, laboratory systems provide researchers with a high degree of control and the ability to manipulate experimental conditions. The wealth of knowledge of microbial genetics and physiology, as well as the amenability of many microbes to genetic engineering, also contribute to making microbial communities attractive model systems. This has become increasingly more appreciated in the scientific community, and recently microbial systems have been used for the investigation of behavioral ecology (103-105), population dynamics (106, 107) and the relationship between community composition and function (108).

Objectives of this thesis

A much used approach to population dynamic studies is the analysis of abundance time series (109, 110). Temporal population data are, however, a scarce resource in microbiological science, and even though various culture-independent techniques may be useful for collecting this sort of data, in general, such methods can often be intensive in terms of cost and workload, or they may have poor quantitative properties. Especially in the case of microbes, the temporal scale on which ecological phenomena may be expected to occur necessitates frequent enumeration of the various sub-populations within a community. An explicit goal of the work presented in this thesis was the development of a quick and inexpensive method for simultaneous abundance monitoring of several bacterial species or strains in an experimental community setting.

A second objective of this study was the collection and analysis of bacterial time series data, within a general ecological framework. This part of the work had a specific focus on bacteria residing in the gastrointestinal tract of animals, using different experimental approaches. Due their importance in health issues these microbes have been the objects of intensive study, yet very little is known about how the community structure of the gut microflora is influenced by bacterial population dynamic phenomena.

Two bacterial species have received special attention in the experiments presented here. *Campylobacter jejuni* is the globally dominant cause of food-borne diarrhoeal

disease, frequently entering the food-chain through infected poultry meat (111, 112). *Clostridium perfringens* is a bacterium which is often part of the normal human gut microbiota. It is also a notorious pathogen, causing diseases like gas gangrene, necrotic enteritis and diarrhoeal food-poisoning (113, 114), as well as being implicated in sudden infant death syndrome (SIDS) (115, 116).

METHODS

Direct DNA sequencing of genetic mixtures

Sanger sequencing of DNA using fluorescence labeled terminator nucleotides produces characteristic emission spectra, called electropherograms, from which the DNA sequences can be deduced. Prior to fluorescence labeling, the target genetic region is often amplified by PCR. If the target gene is sufficiently conserved, such as the bacterial 16S rRNA gene, PCR primers may be designed so that they amplify across a broad taxonomic range, producing a mixture of amplicons. When such a mixture is used as template for a chain termination fluorescence labeling PCR, and the resulting product run through a sequencing apparatus, multiple fluorescence peaks will be produced in the electropherogram at polymorphic nucleotide positions. Such an electropherogram contains quantitative information about the different DNA species contributing to the complex spectrum. As is shown in paper I, this information can be extracted using multivariate statistical techniques.

Multivariate analysis of complex electropherograms

A regular DNA sequence electropherogram contains a large number of varibles (typically several thousand fluorescence readings for a sequence of 100 nucleotides). In order to analyze data of this kind special statistical tools are needed. We developed two separate, but related approaches for the quantitative analysis of complex DNA sequence electropherograms.

Using multiple linear regression (MLR) according to the linear mixture model (117), a complex spectrum is modeled as a linear combination of its constituent pure

spectra (unit spectra). The coefficients estimated from this regression are then interpreted as the relative abundances of the species contributing to the mixture.

The second approach uses partial least squares regression (PLSR) for predicting species abundances from complex electropherograms. This is a data compression technique which is based on extracting latent variables from large data sets (117). The use of orthogonal latent variables effectively eliminates the problem of near collinearity which is often encountered in spectral data (118), and the PLSR algorithm is such that the extracted variables are optimal for quantitative prediction.

Experimental systems

We used three different experimental systems for producing bacterial community time series data, using the methods described in paper I.

In paper II, we took an in vivo approach to the investigation of multiple strain *C.jejuni* infections, using broiler chickens as an infection model. Estimation of relative strain abundances was coupled with the determination of total *C.jejuni* loads by real-time PCR, and the experimental design included groups of chickens with spontaneous or induced background gut microfloras.

In paper III, we used a batch culture fermentation setup, with three species of common gut bacteria. In this experiment, collection of close interval time series data was coupled with on-line monitoring of the chemical environment in the culture by Fourier-transform infrared (FT-IR) spectroscopy.

In paper IV, we used a chemostat with four species of gut bacteria, including *C.perfringens*, growing together under low dilution rates with fresh medium. Relative species abundances were surveyed on an hourly basis, and pH and reduction potential in the growth chamber were logged continuously. Total bacterial abundance was represented as optical densities at 600nm.

Time series analysis

The experimental procedure used in paper II did not result in time series data in the strict sense, since the chickens were slaughtered prior to sampling of caecal material for determination of *C.jejuni* abundances. The samples were, however, collected sequentially

from different chickens over a period several weeks, and samples taken at different time points and from different experimental groups, and strain compositions were compared using classical statistical methods.

The time series from the in vitro community experiments were analyzed using generalized additive models (GAMs), as implemented I the R package 'mgcv' (119, 120). This is a non-parametric regression technique which does not require an *a priori* specification of the functional form between response and predictor variables. The GAM procedure consists of fitting smooth additive functions to each covariate term included in the model structure, with the smooth functions being weighted sums of basis cubic spline functions. The weights are chosen according to the generalized cross validation (GCV) criterion (119), which balances model fit with model complexity. This technique is well suited for modeling nonlinear relationships, and has often been used in the analysis of ecological time series (121-124).

RESULTS AND DISCUSSION

An initial objective of this study was the development of a high-throughput methodology for easy quantification of multiple microbial species or strains in complex experimental samples. The direct sequencing approach requires, besides a robust and unbiased DNA extraction protocol (125, 126), only PCR amplification with a suitable primer set, followed by standard Sanger DNA sequencing. Multivariate statistical analysis, with methodologies borrowed from chemometric science, was shown to be effective and accurate for determining relative abundances of genotypes in mixed species samples. The two different analytical approaches (MLR and PLSR) were both found to perform very well. The MLR approach has the advantage of not requiring any prior calibration of a prediction model, making it quite fast and easy to implement. It does, however, presuppose a linear relationship between the complex electropherogram and the corresponding unit spectra, and this presupposition should be validated for any given experimental setting. For computing a PLSR prediction model, calibration data, in the form of a designed set of known mixtures, are needed. Thus, this method involves some additional labor relative to the MLR approach. It does, however, tend to give more

accurate predictions, accommodating nonlinearities in the relationship between the complex electropherograms and relative species abundances.

The methods described in paper I can, in principle, be applied to any defined mixture of DNA molecules, provided that the different species in the mixture can be sequenced with a common, unbiased primer. However, there are special considerations to be taken in the PCR step intermediate between DNA extraction and sequencing. When amplifying a target gene from a mixed template sample, the quantitative relationship between the amplified fragments will only be representative of the initial sample if amplification efficiency is the same for all genotypes present. PCR amplification bias may be introduced by factors such as differential primer specificity (127), template ratios and number of PCR cycles (128), target gene copy number and genome size (129). Such pitfalls need to be considered when performing mixed template PCRs.

The direct sequencing/multivariate analysis method described above was used for data collection in three different experimental settings, illustrating applications of the methodology.

Paper II describes the co-infection kinetics of seven *C.jejuni* strains in a chicken model. Enumeration of the different strains was carried out, in replicates, from cecal material at six time points over the course of 36 days. Quantification was done using the MLR approach on the citrate synthase (*gltA*) gene, which is strain polymorphic in *C.jejuni*. Half of the chickens had been treated with a preparation of mature chicken gut microflora (Broilact). This treatment was found to have a pronounced effect on the establishment of the microflora in treated vs. non-treated birds. It was, however, not found to have a significant effect on the course of *C.jejuni* infections. Two strains were found to be dominantly invasive, and these were the same across the two treatment groups. Furthermore, in both treatment groups we observed an abrupt shift in predominance of the two strains, and this shift occurred significantly earlier in treated birds. The developed hypothesis stated that, in analogy to a model developed for the Plasmodium parasite (130, 131), the infection dynamics are determined by the host through adaptive immune responses. In this model, the host launches a strong, specific immune response against a dominant infecting strain, while the same response suppresses the other infecting strains in a less specific manner. When the immune response causes

the dominant strain to collapse, inter-strain competition is temporarily relaxed, allowing the outgrowth of a secondary strain, against which a specific immune response does not yet exist.

The description of *C.jejuni* co-infections demonstrates the application of the direct sequencing/multivariate analysis approach on an *in vivo* system, against a very complex background microflora. This illustrates the point that the methodology can be used to look at any organismal group, at the taxonomic level of interest, as long as pertinent genetic markers exist, allowing the relevant DNA sequence information to be separated from the background.

In papers III and IV we used in vitro systems in order to look at interactions between members of the gastrointestinal microbiota. Temporal population data were, in both cases, collected by application of the direct sequencing/multivariate analysis approach, using PLSR models. Since these experiments involved species belonging to distantly related lineages, we used the 16S rRNA gene as a marker for quantification at the species level.

Paper III illustrates the use of time series analysis on data from a 3 species (*Escherichia coli*, *Bacteroides uniformis* and *Lactobacillus salivarius*) system in a batch culture. We fitted GAMs to each series of population data from 30 hours of fermentative growth, including pH as a covariate in the models. This approach allowed us to relate the growth of each species to relative population abundance data and pH at consecutive time points, providing a description of dynamics and interactions in the system. Interpretation was aided by the availability of chemical data from the co-culture, as measured by on-line FT-IR spectroscopy. These data supplied us with information on changing ratios of primary fermentative metabolites in the growth chamber. We were able to identify and describe interactions both within and between species, demonstrating that extensive dynamics may be detected in short term mixed bacterial cultures. Our approach also demonstrated the usefulness of taking an ecological approach to bacterial community analysis, and that such communities may be analyzed within the same conceptual framework as plant and animal systems.

In paper IV we used a chemostat in order to study a four species system of gut bacteria. The bacterial species were selected to represent main divisions of the human

gastrointestinal microflora. The human distal gut has been found to be dominated by the phyla Firmicutes and Bacteroidetes (14), which, in our system, were represented by *Clostridium perfringens* and *Bacteroides thetaiotaomicron*, respectively. The Actinobacteria and Proteobacteria, two other bacterial divisions found in the human GI tract, were represented by *Bifidobacterium longum* and *Escherichia coli*, respectively. *B.thetaiotaomicron* is the most predominant species in the human gut (14), and it is a symbiont providing several important physiological functions to its host (132, 133). *C.perfringens* is a widespread and potentially lethal pathogen commonly found in the human gut microbiota (114). These two species were also found to dominate in our experimental chemostat system, with *E.coli* and *B.longum* occurring at much lower densities. The fact that this simple model system spontaneously adopted a community structure similar to that of the adult human distal gut, at the division level, suggests the possibility that inter-bacterial interactions may play a role in establishing the mature GI microflora. We analyzed 80 hours of time series data from the chemostat by fitting GAMs to each population series. This analysis identified a network of interactions, showing that *B.thetaiotaomicron* was a key species in the regulation of the system's dynamic structure. In particular, *B.thetaiotaomicron* was found to check the growth of *C.perfringens*. During this experiment pH and RedOx potential were also included as covariates in the time series analysis, and the results suggested ways in which these parameters could be used for manipulating the system, including the prevalence of *C.perfringens*.

In infants of less than one year of age the colonization of the intestinal system is seemingly chaotic in terms of composition at the division level (52), and it is likely that host selection plays a lesser role in shaping the early gut microflora due to the absence of a mature immune system. Thus, the dynamics of the infant gut microbiota may, to a larger extent, be dictated by intrinsic mechanisms. Toxigenic *C.perfringens* have been implicated in infant mortality as the causative agent of SIDS (115, 116, 134). The ecological approach to gut bacterial community analysis allowed us to identify the kinds of interactions that contribute to shaping the structural dynamics of experimental systems, be that intrinsic, density-dependent interactions, or environmental interactions. This type of knowledge could be used to control complex microbial systems, like that of the infant

gut, leading to prevention strategies against diseases like SIDS, and the promotion of a healthy and stable GI microbiota.

FUTURE PERSPECTIVES

We have developed a complete methodology for the analysis of community dynamics in experimental microbial systems, drawing on techniques from microbiology, molecular genetics, chemometrics and general ecology. Microbial ecology is a scientific discipline in which integrated approaches are very much required. General ecological theory and methodology has traditionally not played a prominent role in microbial ecology, but this trend is starting to change (135), giving great promise for future discoveries.

Ecological control of the gut microflora

The manipulation of the gastrointestinal microbiota has been attempted both in humans and animals using probiotic agents that presumably confer health benefits upon the recipient (136-139), e.g. through the principle of competitive exclusion (140, 141). A more advanced knowledge of interactions between gut bacteria, as well as between the normal gut flora and opportunist pathogenic colonizers, should lead to a more guided approach toward the development of strategies for manipulating this system. The results presented in paper IV point towards such an approach, where the manipulation of chemical parameters may be used to hamper the competitive ability of *C.perfringens*, either directly or through promoting the growth of an innocuous competing species. This should be tried experimentally using both *in vitro* and *in vivo* systems.

Co-infection dynamics

The extent to which pathogens colonize susceptible hosts as multiple strain/species consortia is not known, but it seems likely that this is not an uncommon scenario (142-151). Such infections may lead to within-host infections dynamics determined by density-dependent interactions between the pathogenic strains/species, as well as density-dependent regulation of the pathogen population as a whole elicited by cross-immunity. This mechanism has been proposed for infections with malaria parasites (131), and as shown in paper II, co-infections with multiple *C.jejuni* strains in chickens show a

dynamic pattern commensurable with this model. One implication of this hypothesis is that vaccines or drug therapies neglecting to target all pathogenic strains or species present in co-infections may relax density-dependent regulation of the parasite population, allowing minority sub-populations to expand. Thus, we need to know more about co-infection dynamics across a wide range of pathogens in order to device more effective prevention and therapeutic strategies. The methods presented here provide a possible platform for such studies.

Microbial ecology and environmental management

The realization that only a tiny proportion of the worlds microbes have been described, and that these microbes constitute a key factor in shaping the earth's environment (1, 48, 152) has led to an increased appreciation of the fact that natural bacterial communities are relatively poorly understood in terms of their ecology. Consequently there is a need for increased knowledge of the specific roles of individual microbial taxa and how their interactions with one another work to determine their collective effects on the environment (153). For example, it has been demonstrated that species richness and synergistic interactions are important in determining the environmental services that a bacterial community can provide (108). An important step towards relating bacterial community structure and function to environmental processes, is an increased understanding of temporal aspects of their interactions. A way of achieving this is through the analysis of time series data pertaining both to abiotic and biotic variables. Molecular tools now provide researchers with unprecedented opportunities for surveying changes in microbial communities on various time scales. Ecological analysis of such data, similarly to the approaches demonstrated in this thesis, should yield important new insights into the interplay between the microbial world and the earth's environment, possibly leading to new paradigms in environmental management.

References:

1. Newman DK & Banfield JF (2002) Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. *Science (New York, N.Y* 296(5570):1071-1077.
2. Whitman WB, Coleman DC, & Wiebe WJ (1998) Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences of the United States of America* 95(12):6578-6583.
3. Haygood MG & Distel DL (1993) Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes form unique lineages related to the genus Vibrio. *Nature* 363(6425):154-156.
4. Ruby EG (1996) Lessons from a cooperative, bacterial-animal association: the Vibrio fischeri-Euprymna scolopes light organ symbiosis. *Annu Rev Microbiol* 50:591-624.
5. Shimomura O & Johnson FH (1975) Chemical nature of bioluminescence systems in coelenterates. *Proceedings of the National Academy of Sciences of the United States of America* 72(4):1546-1549.
6. Madigan MT, Parker J, Brock TD, & Martinko JM (2003) *Brock biology of microorganisms* (Prentice Hall, Upper Saddle River, N.J.) 10th Ed pp XXV, 1019, [1058] s.
7. Russell JB & Rychlik JL (2001) Factors that alter rumen microbial ecology. *Science (New York, N.Y* 292(5519):1119-1122.
8. Warnecke F*, et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* 450(7169):560-565.
9. Guarner F & Malagelada JR (2003) Gut flora in health and disease. *Lancet* 361(9356):512-519.
10. Nicholson JK, Holmes E, & Wilson ID (2005) Gut microorganisms, mammalian metabolism and personalized health care. *Nature reviews* 3(5):431-438.
11. Zoetendal EG, Vaughan EE, & de Vos WM (2006) A microbial world within us. *Molecular microbiology* 59(6):1639-1650.

12. Savage DC (1977) Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* 31:107-133.
13. Ley RE, Peterson DA, & Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124(4):837-848.
14. Eckburg PB*, et al.* (2005) Diversity of the human intestinal microbial flora. *Science (New York, N.Y* 308(5728):1635-1638.
15. Zoetendal EG, Collier CT, Koike S, Mackie RI, & Gaskins HR (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. *The Journal of nutrition* 134(2):465-472.
16. Falk PG, Hooper LV, Midtvedt T, & Gordon JI (1998) Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* 62(4):1157-1170.
17. Cash HL, Whitham CV, Behrendt CL, & Hooper LV (2006) Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science (New York, N.Y* 313(5790):1126-1130.
18. Kelly D, Conway S, & Aminov R (2005) Commensal gut bacteria: mechanisms of immune modulation. *Trends in immunology* 26(6):326-333.
19. Mazmanian SK, Liu CH, Tzianabos AO, & Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122(1):107-118.
20. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, & Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118(2):229-241.
21. Stappenbeck TS, Hooper LV, & Gordon JI (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America* 99(24):15451-15455.
22. Hooper LV, Midtvedt T, & Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual review of nutrition* 22:283-307.

23. Li M*, et al.* (2008) Symbiotic gut microbes modulate human metabolic phenotypes. *Proceedings of the National Academy of Sciences of the United States of America* 105(6):2117-2122.
24. Pryde SE, Duncan SH, Hold GL, Stewart CS, & Flint HJ (2002) The microbiology of butyrate formation in the human colon. *FEMS microbiology letters* 217(2):133-139.
25. Kelly D, King T, & Aminov R (2007) Importance of microbial colonization of the gut in early life to the development of immunity. *Mutation research* 622(1-2):58-69.
26. Penders J, Stobberingh EE, van den Brandt PA, & Thijs C (2007) The role of the intestinal microbiota in the development of atopic disorders. *Allergy* 62(11):1223-1236.
27. Scanlan PD*, et al.* (2008) Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environmental microbiology* 10(3):789-798.
28. Frank DN*, et al.* (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America* 104(34):13780-13785.
29. Ley RE, Turnbaugh PJ, Klein S, & Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* 444(7122):1022-1023.
30. Turnbaugh PJ*, et al.* (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444(7122):1027-1031.
31. Sogin SJ, Sogin ML, & Woese CR (1971) Phylogenetic measurement in procaryotes by primary structural characterization. *Journal of molecular evolution* 1(1):173-184.
32. Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* 74(11):5088-5090.
33. Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR, & Stahl DA (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol* 40:337-365.

34. Stahl DA, Lane DJ, Olsen GJ, & Pace NR (1984) Analysis of Hydrothermal Vent-Associated Symbionts by Ribosomal RNA Sequences. *Science (New York, N.Y* 224(4647):409-411.
35. Stahl DA, Lane DJ, Olsen GJ, & Pace NR (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl Environ Microbiol* 49(6):1379-1384.
36. Saiki RK*, et al.* (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (New York, N.Y* 239(4839):487-491.
37. Giovannoni SJ, Britschgi TB, Moyer CL, & Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 345(6270):60-63.
38. Britschgi TB & Giovannoni SJ (1991) Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Applied and environmental microbiology* 57(6):1707-1713.
39. Schmidt TM, DeLong EF, & Pace NR (1991) Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *Journal of bacteriology* 173(14):4371-4378.
40. Weller R, Bateson MM, Heimbuch BK, Kopczynski ED, & Ward DM (1992) Uncultivated cyanobacteria, Chloroflexus-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. *Applied and environmental microbiology* 58(12):3964-3969.
41. DeLong EF (1992) Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America* 89(12):5685-5689.
42. Fuhrman JA, McCallum K, & Davis AA (1992) Novel major archaebacterial group from marine plankton. *Nature* 356(6365):148-149.
43. Ward DM, Weller R, & Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345(6270):63-65.
44. Stackebrandt E, Liesack W, & Goebel BM (1993) Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *Faseb J* 7(1):232-236.

45. Keller M & Zengler K (2004) Tapping into microbial diversity. *Nature reviews* 2(2):141-150.
46. Pace NR (1997) A molecular view of microbial diversity and the biosphere. *Science (New York, N.Y* 276(5313):734-740.
47. Rappe MS & Giovannoni SJ (2003) The uncultured microbial majority. *Annu Rev Microbiol* 57:369-394.
48. Torsvik V, Ovreas L, & Thingstad TF (2002) Prokaryotic diversity--magnitude, dynamics, and controlling factors. *Science (New York, N.Y* 296(5570):1064-1066.
49. Woese CR (1987) Bacterial evolution. *Microbiological reviews* 51(2):221-271.
50. Sun HY, Deng SP, & Raun WR (2004) Bacterial community structure and diversity in a century-old manure-treated agroecosystem. *Appl Environ Microbiol* 70(10):5868-5874.
51. Weber S, Stubner S, & Conrad R (2001) Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. *Appl Environ Microbiol* 67(3):1318-1327.
52. Palmer C, Bik EM, Digiulio DB, Relman DA, & Brown PO (2007) Development of the Human Infant Intestinal Microbiota. *PLoS Biol* 5(7):e177.
53. Muyzer G, de Waal EC, & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59(3):695-700.
54. Zoetendal EG, Akkermans AD, & De Vos WM (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* 64(10):3854-3859.
55. Zoetendal EG*, et al.* (2002) Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl Environ Microbiol* 68(7):3401-3407.
56. Lee DH, Zo YG, & Kim SJ (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Appl Environ Microbiol* 62(9):3112-3120.

57. Peters S, Koschinsky S, Schwieger F, & Tebbe CC (2000) Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. *Applied and environmental microbiology* 66(3):930-936.
58. Kaplan CW, Astaire JC, Sanders ME, Reddy BS, & Kitts CL (2001) 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed Lactobacillus acidophilus NCFM. *Appl Environ Microbiol* 67(4):1935-1939.
59. Liu WT, Marsh TL, Cheng H, & Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* 63(11):4516-4522.
60. Fisher MM & Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl Environ Microbiol* 65(10):4630-4636.
61. Ranjard L*, et al.* (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Appl Environ Microbiol* 67(10):4479-4487.
62. Bond PL & Banfield JF (2001) Design and Performance of rRNA Targeted Oligonucleotide Probes for in Situ Detection and Phylogenetic Identification of Microorganisms Inhabiting Acid Mine Drainage Environments. *Microbial ecology* 41(2):149-161.
63. Li S, Spear RN, & Andrews JH (1997) Quantitative fluorescence in situ hybridization of Aureobasidium pullulans on microscope slides and leaf surfaces. *Appl Environ Microbiol* 63(8):3261-3267.
64. Schrenk MO, Edwards KJ, Goodman RM, Hamers RJ, & Banfield JF (1998) Distribution of thiobacillus ferrooxidans and leptospirillum ferrooxidans: implications for generation of acid mine drainage. *Science (New York, N.Y* 279(5356):1519-1522.
65. Guschin DY*, et al.* (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Appl Environ Microbiol* 63(6):2397-2402.

66. Wagner M, Smidt H, Loy A, & Zhou J (2007) Unravelling microbial communities with DNA-microarrays: challenges and future directions. *Microbial ecology* 53(3):498-506.
67. Rudi K, Flateland SL, Hanssen JF, Bengtsson G, & Nissen H (2002) Development and evaluation of a 16S ribosomal DNA array-based approach for describing complex microbial communities in ready-to-eat vegetable salads packed in a modified atmosphere. *Appl Environ Microbiol* 68(3):1146-1156.
68. Taroncher-Oldenburg G, Griner EM, Francis CA, & Ward BB (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. *Appl Environ Microbiol* 69(2):1159-1171.
69. Wu L*, et al.* (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. *Appl Environ Microbiol* 67(12):5780-5790.
70. Acinas SG*, et al.* (2004) Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* 430(6999):551-554.
71. Li H, Yang SZ, Mu BZ, Rong ZF, & Zhang J (2007) Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. *FEMS microbiology ecology* 60(1):74-84.
72. McCaig AE, Glover LA, & Prosser JI (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Appl Environ Microbiol* 65(4):1721-1730.
73. Ohkuma M & Kudo T (1996) Phylogenetic diversity of the intestinal bacterial community in the termite Reticulitermes speratus. *Appl Environ Microbiol* 62(2):461-468.
74. Sanger F, Nicklen S, & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74(12):5463-5467.
75. Sjoling S & Cowan DA (2003) High 16S rDNA bacterial diversity in glacial meltwater lake sediment, Bratina Island, Antarctica. *Extremophiles* 7(4):275-282.

76. Ley RE*, et al.* (2006) Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. *Appl Environ Microbiol* 72(5):3685-3695.
77. Huang XC, Quesada MA, & Mathies RA (1992) DNA sequencing using capillary array electrophoresis. *Analytical chemistry* 64(18):2149-2154.
78. Kambara H & Takahashi S (1993) Multiple-sheathflow capillary array DNA analyser. *Nature* 361(6412):565-566.
79. Kim UJ, Shizuya H, de Jong PJ, Birren B, & Simon MI (1992) Stable propagation of cosmid sized human DNA inserts in an F factor based vector. *Nucleic acids research* 20(5):1083-1085.
80. Shizuya H*, et al.* (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proceedings of the National Academy of Sciences of the United States of America* 89(18):8794-8797.
81. Stein JL, Marsh TL, Wu KY, Shizuya H, & DeLong EF (1996) Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *Journal of bacteriology* 178(3):591-599.
82. DeLong EF*, et al.* (2006) Community genomics among stratified microbial assemblages in the ocean's interior. *Science (New York, N.Y* 311(5760):496-503.
83. Rondon MR*, et al.* (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl Environ Microbiol* 66(6):2541-2547.
84. Tringe SG*, et al.* (2005) Comparative metagenomics of microbial communities. *Science (New York, N.Y* 308(5721):554-557.
85. Tyson GW*, et al.* (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428(6978):37-43.
86. Venter JC*, et al.* (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science (New York, N.Y* 304(5667):66-74.

87. Braslavsky I, Hebert B, Kartalov E, & Quake SR (2003) Sequence information can be obtained from single DNA molecules. *Proceedings of the National Academy of Sciences of the United States of America* 100(7):3960-3964.
88. Margulies M*, et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437(7057):376-380.
89. Ronaghi M, Uhlen M, & Nyren P (1998) A sequencing method based on real-time pyrophosphate. *Science (New York, N.Y* 281(5375):363, 365.
90. Shendure J*, et al.* (2005) Accurate multiplex polony sequencing of an evolved bacterial genome. *Science (New York, N.Y* 309(5741):1728-1732.
91. Huber JA*, et al.* (2007) Microbial population structures in the deep marine biosphere. *Science (New York, N.Y* 318(5847):97-100.
92. Sogin ML*, et al.* (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences of the United States of America* 103(32):12115-12120.
93. Dinsdale EA*, et al.* (2008) Functional metagenomic profiling of nine biomes. *Nature* 452(7187):629-632.
94. May RM (1976) *Theoretical Ecology: Principles and Applications* (Blackwell Scientific Publications, Oxford).
95. Gause GF (1932) Experimental Studies on the Struggle for Existence: I. Mixed Population of Two Species of Yeast. *J Exp Biol* 9(4):389-402.
96. Gause GF (1935) Experimental Demonstration of Volterra's Periodic Oscillations in the Numbers of Animals. *J Exp Biol* 12(1):44-48.
97. Jessup CM*, et al.* (2004) Big questions, small worlds: microbial model systems in ecology. *Trends Ecol Evol* 19(4):189-197.
98. Prosser JI*, et al.* (2007) The role of ecological theory in microbial ecology. *Nature reviews* 5(5):384-392.
99. Sogin ML*, et al.* (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences of the United States of America* 103(32):12115-12120.
100. Hansen SK, Rainey PB, Haagensen JA, & Molin S (2007) Evolution of species interactions in a biofilm community. *Nature* 445(7127):533-536.

101. Rainey PB & Rainey K (2003) Evolution of cooperation and conflict in experimental bacterial populations. *Nature* 425(6953):72-74.
102. Cooper VS & Lenski RE (2000) The population genetics of ecological specialization in evolving Escherichia coli populations. *Nature* 407(6805):736-739.
103. Diggle SP, Griffin AS, Campbell GS, & West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450(7168):411-414.
104. Griffin AS, West SA, & Buckling A (2004) Cooperation and competition in pathogenic bacteria. *Nature* 430(7003):1024-1027.
105. MacLean RC & Gudelj I (2006) Resource competition and social conflict in experimental populations of yeast. *Nature* 441(7092):498-501.
106. Becks L, Hilker FM, Malchow H, Jurgens K, & Arndt H (2005) Experimental demonstration of chaos in a microbial food web. *Nature* 435(7046):1226-1229.
107. Beninca E*, et al.* (2008) Chaos in a long-term experiment with a plankton community. *Nature* 451(7180):822-825.
108. Bell T, Newman JA, Silverman BW, Turner SL, & Lilley AK (2005) The contribution of species richness and composition to bacterial services. *Nature* 436(7054):1157-1160.
109. Bjornstad ON & Grenfell BT (2001) Noisy clockwork: time series analysis of population fluctuations in animals. *Science (New York, N.Y* 293(5530):638-643.
110. Turchin P & Taylor AD (1992) Complex Dynamics in Ecological Time-Series. *Ecology* 73(1):289-305.
111. Champion OL*, et al.* (2005) Comparative phylogenomics of the food-borne pathogen Campylobacter jejuni reveals genetic markers predictive of infection source. *Proceedings of the National Academy of Sciences of the United States of America* 102(44):16043-16048.
112. Parkhill J*, et al.* (2000) The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. *Nature* 403(6770):665-668.
113. Hatheway CL (1990) Toxigenic clostridia. *Clinical microbiology reviews* 3(1):66-98.

114. Shimizu T*, et al.* (2002) Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. *Proceedings of the National Academy of Sciences of the United States of America* 99(2):996-1001.
115. Lindsay JA*, et al.* (1993) Clostridium-Perfringens Type-a Cytotoxic-Enterotoxin(S) as Triggers for Death in the Sudden-Infant-Death-Syndrome - Development of a Toxico-Infection Hypothesis. *Current Microbiology* 27(1):51-59.
116. Murrell WG, Stewart BJ, O'Neill C, Siarakas S, & Kariks S (1993) Enterotoxigenic bacteria in the sudden infant death syndrome. *Journal of medical microbiology* 39(2):114-127.
117. Martens H & Næs T (1989) *Multivariate calibration* (Wiley, Chichester) pp XVII, 419 s.
118. Naes T & Mevik B (2001) Understanding the collinearity problem in regression and discriminant analysis. *J CHEMOMETR* 15(4):413-426.
119. Wood SN (2000) Modelling and smoothing parameter estimation with multiple quadratic penalties. *Journal of the Royal Statistical Society Series B-Statistical Methodology* 62:413-428.
120. Wood SN (2006) *Generalized additive models : an introduction with R* (Chapman & Hall/CRC, Boca Raton) pp xvii, 391 s.
121. Ciannelli L, Bailey KM, Chan KS, Belgrano A, & Stenseth NC (2005) Climate change causing phase transitions of walleye pollock (Theragra chalcogramma) recruitment dynamics. *Proceedings of the Royal Society B-Biological Sciences* 272(1573):1735-1743.
122. Ciannelli L, Chan KS, Bailey KM, & Stenseth NC (2004) Nonadditive effects of the environment on the survival of a large marine fish population. *Ecology* 85(12):3418-3427.
123. Stenseth NC*, et al.* (2006) Seasonal plankton dynamics along a cross-shelf gradient. *Proceedings of the Royal Society B-Biological Sciences* 273(1603):2831-2838.

124. Stige LC*, et al.* (2006) The effect of climate variation on agro-pastoral production in Africa. *Proceedings of the National Academy of Sciences of the United States of America* 103(9):3049-3053.
125. Skanseng B, Kaldhusdal M, & Rudi K (2006) Comparison of chicken gut colonisation by the pathogens Campylobacter jejuni and Clostridium perfringens by real-time quantitative PCR. *Molecular and cellular probes* 20(5):269-279.
126. von Wintzingerode F, Gobel UB, & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS microbiology reviews* 21(3):213-229.
127. Polz MF & Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* 64(10):3724-3730.
128. Suzuki MT & Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62(2):625-630.
129. Farrelly V, Rainey FA, & Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol* 61(7):2798-2801.
130. Bruce MC & Day KP (2002) Cross-species regulation of malaria parasitaemia in the human host. *Current opinion in microbiology* 5(4):431-437.
131. Bruce MC*, et al.* (2000) Cross-species interactions between malaria parasites in humans. *Science (New York, N.Y* 287(5454):845-848.
132. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, & Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science (New York, N.Y* 307(5717):1915-1920.
133. Xu J & Gordon JI (2003) Inaugural Article: Honor thy symbionts. *Proceedings of the National Academy of Sciences of the United States of America* 100(18):10452-10459.
134. Lindsay JA (1996) Clostridium perfringens type A enterotoxin (CPE): more than just explosive diarrhea. *Critical reviews in microbiology* 22(4):257-277.
135. Head IM & Prosser JI (2007) Microorganisms, macroorganisms and ecology. *FEMS microbiology ecology* 62(2):133-232.

136. Balcazar JL*, et al.* (2006) The role of probiotics in aquaculture. *Veterinary microbiology* 114(3-4):173-186.
137. Sheil B, Shanahan F, & O'Mahony L (2007) Probiotic effects on inflammatory bowel disease. *The Journal of nutrition* 137(3 Suppl 2):819S-824S.
138. Gerard P, Brezillon C, Quere F, Salmon A, & Rabot S (2008) Characterization of cecal microbiota and response to an orally administered lactobacillus probiotic strain in the broiler chicken. *Journal of molecular microbiology and biotechnology* 14(1-3):115-122.
139. Reid G, Jass J, Sebulsky MT, & McCormick JK (2003) Potential uses of probiotics in clinical practice. *Clinical microbiology reviews* 16(4):658-672.
140. Davies TJ (2006) Evolutionary ecology: when relatives cannot live together. *Curr Biol* 16(16):R645-647.
141. Nurmi E, Nuotio L, & Schneitz C (1992) The competitive exclusion concept: development and future. *International journal of food microbiology* 15(3-4):237-240.
142. Gray JT*, et al.* (2001) Recognition of Yersinia enterocolitica multiple strain infection in twin infants using PCR-based DNA fingerprinting. *Journal of applied microbiology* 90(3):358-364.
143. Maitland K, Williams TN, & Newbold CI (1997) Plasmodium vevax and P. falciparum: Biological interactions and the possibility of cross-species immunity. *Parasitology today (Personal ed* 13(6):227-231.
144. Mehlotra RK*, et al.* (2000) Random distribution of mixed species malaria infections in Papua New Guinea. *The American journal of tropical medicine and hygiene* 62(2):225-231.
145. Paul RE*, et al.* (1995) Mating patterns in malaria parasite populations of Papua New Guinea. *Science (New York, N.Y* 269(5231):1709-1711.
146. Richardson JF*, et al.* (2001) Coinfection with Campylobacter species: an epidemiological problem? *Journal of applied microbiology* 91(2):206-211.
147. Salama NR*, et al.* (2007) Genetic analysis of Helicobacter pylori strain populations colonizing the stomach at different times postinfection. *Journal of bacteriology* 189(10):3834-3845.

148. Seinost G*, et al.* (1999) Infection with multiple strains of Borrelia burgdorferi sensu stricto in patients with Lyme disease. *Archives of dermatology* 135(11):1329-1333.
149. Warren RM*, et al.* (2004) Patients with active tuberculosis often have different strains in the same sputum specimen. *American journal of respiratory and critical care medicine* 169(5):610-614.
150. Slutsky AM*, et al.* (1994) Polyclonal infections due to Mycobacterium avium complex in patients with AIDS detected by pulsed-field gel electrophoresis of sequential clinical isolates. *Journal of clinical microbiology* 32(7):1773-1778.
151. Pitt TL, Trakulsomboon S, & Dance DA (2007) Recurrent melioidosis: possible role of infection with multiple strains of Burkholderia pseudomallei. *Journal of clinical microbiology* 45(2):680-681.
152. Kasting JF & Siefert JL (2002) Life and the evolution of Earth's atmosphere. *Science (New York, N.Y* 296(5570):1066-1068.
153. Paerl HW & Steppe TF (2003) Scaling up: the next challenge in environmental microbiology. *Environmental microbiology* 5(11):1025-1038.